Specification

Antitumor Protein and Gene Encoding Same

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to an antitumor protein and an nucleotide sequence encoding the same.

Background Art

Various studies have been conducted on antitumor substances found in edible mushrooms.

For example, polysaccharides and glycoprotein from mushrooms which have antitumor activity are disclosed in Japanese Patent Laid-open Publication Nos.61214/1977, 74797/1980, 293923/1986, 70362/1993 and 80699/1994, Japanese Patent Publication Nos. 47518/1986, 47519/1986 and 26172/1991. It is also reported that mushrooms are found to have antitumor activity when administered.

However, there has been no report on an amino acid sequence of an antitumor protein derived from Tricholoma matsutake which directly kills a tumor cell and on a gene encoding said protein.

SUMMARY OF THE INVENTION

The inventors now have purified an antitumor protein derived from Tricholoma matsutake and determined an amino acid sequence as well as a cDNA sequence encoding the protein. Further, the inventors have successfully purified the cDNA sequence and obtained a recombinant antitumor protein expressed in E. coli which is transformed by introducing a vector comprising the cDNA sequence. The present invention is based on these findings.

Thus, an object of the present invention is to provide an antitumor protein, a fragment of said protein, a nucleotide molecule encoding said protein, a vector comprising said molecule, a host cell transformed by said vector, a process for preparing said protein, and an antibody against said protein.

The protein according to the present invention comprises

(a) an amino acid sequence of SEQ ID No.1, or

(b) a modified amino acid sequence of SEQ ID No.1 which has antitumor activity wherein one or more amino acids are added and/or inserted into the amino acid sequence of SEQ ID No.1 and/or one or more amino acids in the amino acid sequence of SEQ ID No.1 are substituted and/or deleted.

The protein according to the present invention is useful as an antitumor agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the structure of plasmid vector pTS18.

Figure 2 illustrates the deletion of the TTM gene. Dashed lines denote a deleted area.

DETAILED DESCRIPTION OF THE INVENTION

Protein

The protein according to the present invention comprises the amino acid sequence of SEQ ID No.1. A protein consisting of the amino acid sequence of SEQ ID No.1 has antitumor activity as described in examples.

Examples of the proteins according to the present invention include those consisting of a modified amino acid sequence of SEQ ID No.1 which has antitumor activity wherein one or more amino acids are added and/or inserted into the amino acid sequence of SEQ ID NO.1 and/or one or more amino acids in the amino acid sequence of SEQ ID NO.1 are substituted and/or deleted. The terms "addition", "insertion", "substitution" and "deletion" refer to those which do not damage the antitumor activity of the protein consisting of the amino acid sequence of SEQ ID NO.1. The numbers of modifications such as additions, insertions, substitutions and deletions may be in the range between 1 and 8.

An addition, insertion, substitution or deletion may be introduced into an amino acid sequence in accordance with, for example, Molecular Cloning (A laboratory manual),

second edition, Cold Spring Harbor Laboratory Press, Vol. 2, Chap. 15 (1989); Botstein, D. et al., Science, 229:1193 (1985); Craik, C.S., Bio. Techniques, 3:12 (1985); Itakura, K. et al., Annu. Rev. Biochem. 53:323 (1984); Shortle, D. et al., Annu. Rev. Genet. 15:265 (1981); or Smith, M. Annu. Rev. Genet. 19:423 (1985).

The wording "protein which have antitumor activity" as used herein refers to a protein which is evaluated by one skilled in the art to have antitumor activity, for example, a protein which is evaluated to have antitumor activity as tested under the conditions in Example 1 (3).

The molecular weight of the protein consisting of the amino acid sequence of SEQ ID NO.1 is about 65 kDa as measured by SDS-PAGE.

The amino acid sequence of SEQ ID NO.1 can be prepared by expression of the DNA sequence of SEQ ID NO.2 in a bacteria using a common technique. The cDNA sequence can be prepared by screening a cDNA library derived from Tricholoma matsutake using an antibody against the antitumor protein as a probe (see Example 2).

The protein according to the present invention has antitumor activity. Therefore, the protein according to the present invention may be formulated in a pharmaceutical composition which is used in the treatment of tumor such as carcinoma of uterine cervix or corpus uteri, and a variety of cancers caused by abnormal expression of antioncogene p53 or pBR (e.g., carcinoma cutaneum, lung cancer, liver cancer, kidney cancer, and breast cancer).

The pharmaceutical composition according to the present invention may be administered to a mammal including a human perorally or parenterally (e.g., intramuscularly, intravenously, subcutaneously, intrarectally, percutaneously or pernasally) in a form suitable for peroral or parenteral administration. A formulation which directly reaches a target area (e.g., a tablet which dissolves at a specific site, a liniment, or an injection) may be preferably used in the treatment of tumor.

The protein according to the present invention may be formulated in oral drugs (e.g., tablets, capsules, granules, powder, pills, grains, troches) when considering stability of the protein and the drug delivery path; injectable drugs (e.g., for intravenous or intramuscular injection); intrarectal drugs; and soluble or insoluble suppositories depending on its intended use. The pharmaceutical composition in these forms may be prepared by conventional methods with pharmaceutically acceptable vehicles such as bulking agents and fillers; adjuvants such as binding agents, wetting agents, disintegrants, surfactants, lubricants, dispersers, buffering agents, and solution adjuvant; additives such as preservatives, antiseptics, flavouring agents, soothing agents, stabilizers, colouring agents, and sweeting agents. A dose for various treatments may be determined depending on the route of administration as well as the age, sex, and condition of the patient.

Nucleotide Sequence

The present invention provides a nucleotide sequence encoding the protein according to the present invention. Examples of such nucleotide sequences include those comprising all or part of the DNA sequence of SEQ ID NO.2. Other examples of such nucleotide sequences include those comprising all or part of the DNA sequence in SEQ ID NO.2.

As mentioned above, the DNA sequence of SEQ ID NO.2 was obtained from a cDNA library derived from Tricholoma matsutake. This DNA sequence contains an open reading frame of the protein which starts at ATG (1-3) and ends at TAA (1699-1701).

The amino acid sequence determines a number of possible base sequences that encode the amino acid sequence in SEQ ID NO.1.

When the amino acid sequence of the protein according to the present invention is given, a nucleotide sequence encoding the amino acid sequence is easily determined, and a variety of nucleotide sequences encoding the amino acid

sequence of SEQ ID NO: 1 can be selected.

Thus, a nucleotide sequence encoding the protein according to the present invention include DNA sequences which degenarate as a result of the genetic code as to the DNA sequence of SEQ ID No.2 as well as RNA sequences corresponding to the DNA sequences.

The nucleotide sequence according to the present invention may be naturally occurred or obtained by synthesis. It may also be synthesized with a part of a sequence derived from the naturally occurring one. DNAs may typically be obtained by screening a chromosome library or a cDNA library in accordance with conventional methods in the field of genetic engineering, for example, by screening a chromosome library or a cDNA library with an appropriate DNA probe obtained based on information of the partial amino acid sequence. The nucleotide sequence according to the present invention can be prepared, for example, from Tricholoma matsutake cDNA library by using an oligonucleotide encoding a peptide selected from SEQ ID Nos.3-18 as a screening probe.

The nucleotide sequences from nature are not specifically restricted to any sources; but may be derived from Tricholoma matsutake or other sources.

Vectors and Transformed Cells

The present invention provides a vector comprising the nucleotide sequence according to the present invention in such a manner that the vector can be replicable and express the protein encoded by the nucleotide sequence in a host cell. In addition, according to the present invention, we provide a host cell transformed by the vector. There is no other restriction to the host-vector system. It may express proteins fused with other proteins. Examples of an expression system of a fusion protein include those expressing MBP (maltose binding protein), GST (glutathione-S-transferase), HA (hemagglutinin), polyhistidine, myc, and Fas.

Examples of such systems expressing fusion proteins

include those expressing β-galactosidase, glutathione-S-transferase, and luciferase.

Examples of vectors include plasmid vectors (e.g., pBluescript SK(-), pBluescript SK(+), pGEX-4T, pGEX-5T, pRIT2T, pBPV, and pSVK3 (Pharmacia, etc.); ZAP Express, pYEUra3, pMAM, and pOG (Toyobo); pET-11a, b, c, and d, pET-20b, pET-28a, b, and c, and pET-32a and b (Novagen); pQE-10, 16, 30, 40, 50, 60, and 70) (Qiagen); virus vectors (e.g., retrovirus vectors and adenovirus vectors); and liposome vectors (e.g., cationic liposome vectors).

In order to prepare a desired protein in the host cell, the vector according to the present invention may have a sequence which regulate expression of the protein (e.g., a promoter sequence, a terminator sequence, or an enhancer sequence) or markers for selecting a host cell (e.g., a neomycin-resistant gene or a kanamycin-resistant gene). Further, the vector may have the nucleotide sequence according to the present invention in a repeted form (e.g., in a tandem form). Such additional sequences may be introduced into the vector. A host cell may be transformed by the vector by conventional methods.

The vector according to the present invention may be prepared by conventional methods and procedures of the genetic engineering field.

Examples of host cells include E. coli (e.g., SOLR, JM109, XL1-Blue MRF', and BL21(DE3)), yeast cells (e.g., YRG-2), Bacillus subtilis, animal cells (e.g., CHO cells, COS cells, human keratinocytes, COP-5, C127, mouse 3T3 cells, FR3T3, and HB101).

The protein according to the present invention is obtained from the culture by culturing host cells which are transformed as described above in an appropriate medium. Therefore, the present invention provides a process for preparing the protein according to the present invention. Such a process enables mass production of an antitumor protein.

The culture of the transformed host cell and culture

condition may essentially be the same as those for the cell to be used. In addition, the protein according to the present invention may be recovered from the culture medium and purified according to conventional methods, for example, chromatography such as ion exchange chromatography, gel filtration chromatography, and immunoaffinity chromatography

Antibody

The present invention provides an antibody against the protein according to the present invention. The term "antibody" as used herein includes a polyclonal antibody or a monoclonal antibody.

The antibody according to the present invention can be prepared by conventional methods, for example, by injecting the protein of SEQ ID NO.1 or a fragment thereof into an animal (e.g., rabbit, rat or mouse) together with suitable carriers (e.g., Freund's complete and incomplete adjuvants) and then purifying the serum from the animal after a certain period.

Specific reaction (i.e., immuno reaction) of the antibody may be used as an indicator of an antitumor protein. Therefore, the antibody according to the present invention may be used for purifying and screening an antitumor protein.

Examples

The present invention is further illustrated by the following Examples which are not intended as a limitation of the invention.

Example 1 Purification of Antitumor Protein

(1) Purification of Protein

An antitumor protein was purified from commercially available (or wild) fresh Tricholoma matsutake by homogenizing it in accordance with conventional methods and then isolating using purifying procedures such as column chromatography, HPLC, and electrophoresis. The detailed procedure is as follows:

A Tris buffer solution containing NaCl and protease

inhibitor (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 0.1 mM IAA (iodoacetamide), 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) was used for the preliminary elution of the protein, followed by precipitation with ammonium sulfate (90% saturated ammonium sulfate). The precipitate was dialyzed with 25 mM Tris-HCl (pH 7.5) containing 1/10 the above protease inhibitor (PI) to desalt. Then, after DEAE Toyopearl (ion exchange chromatography), concentration of the active fraction, purification through phenyl Sepharose (hydrophobic chromatography), concentration of the active fraction, gel filtration by HPLC (TSK gel G3000SW), the purified protein was finally obtained.

In ion exchange chromatography and hydrophobic chromatography, 25 mM Tris-HCl (pH 7.5) containing PI was used as eluant. For linear concentration gradient, NaCl and $(NH_4)_2SO_4$ were used, respectively. In gel filtration, 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1 M Na_2SO_4 and PI was used as eluant.

The sample obtained by gel filtration with HPLC was analyzed by SDS-PAGE. The protein on the gel, which was transferred to a PVDF membrane and stained with CBB, exhibited a single band (about 65 kDa).

It was found that when Tricholoma matsutake with no freshness was used or when no protease inhibitor was used in purifying procedures, yield and antitumor activity were found to be lower.

Some of the samples were recovered by staining the gel with CBB after SDS-PAGE, and cutting it to extract electrically. These samples were used to determine the amino acid sequence (Example 2).

It was also found that the protein can be purified by affinity chromatography using a column in which the antibody (see (2)) was bound to CNBr-activated Sepharose 6MB resin (Pharmacia).

(2) Polyclonal Antibody

A rabbit was immunized with the protein purified in (1) to prepare antiserum. The procedure is as follows:

The purified protein, 15 μg, was mixed with Freund's complete adjuvant, stirred intensely to emulsion, and subcutaneously injected to the back of a rabbit. After 3 weeks, the rabbit was boosted with 150 μg of the purified protein, which was mixed with Freund's incomplete adjuvant to give emulsion. Then, after 2 weeks, they were directly reboosted using 50 μg of antibody, and blood was collected from its earlobe 1 week later.

Next, 5 ml of antiserum was incubated at 56°C for 30 min, mixed with 5 ml of PBS(-) and the same amount of saturated $(NH_4)_2SO_4$, and maintained still in iced water. After centrifugation, the precipitate was redissolved in sodium phosphate buffer solution and mixed with an additional amount of saturated $(NH_4)_2SO_4$ to a final $(NH_4)_2SO_4$ concentration of 20%. After centrifugation, the supernatant was recovered and mixed with an additional amount of saturated $(NH_4)_2SO_4$ to a final $(NH_4)_2SO_4$ concentration of 33%. After centrifugation, the precipitate was recovered and redissolved. It was then dialyzed and desalted, followed by ion exchange chromatography (DE52 resin), to give an IgG fraction.

(3) Antitumor Activity Test

Lethal activity was investigated on cells which had been transformed by simian virus 40 (SV40) and human papiloma virus (HPV) which were known to cause malignant alteration. More specifically, antitumor activity was estimated with lethal activity. When the protein purified in (1) above was given to the cells, the quantity of the tested protein necessary for 50% fatal activity of total cells was 10 ng/ml in SVT2 cells (transformed SV40 cells), 100 ng/ml in A31 cells (transformed SV40 cells), and 15-20 ng/ml in human preputial cells (transformed HPV16 cells).

Example 2 cDNA Cloning and Sequencing

The amino acid sequence at the N-terminal of the

protein purified in Example 1 was determined (SEQ ID NOS.3 and 4) using a protein sequencer (Hewlett-Packard).

Also, the protein obtained in Example 1 was digested using lysyl endnuclease to give a number of peptide fragments. Among them, the amino acid sequences of 14 peptide fragments were determined (SEQ ID NOS.5-18).

On the other hand, Tricholoma matsutake mRNA was purified with oligo-dT Latex (oligo-dT particles; Takara), then with STRATAGENE ZAP-cDNA Synthesis Kit (available from Toyobo), to synthesize cDNA. After synthesized, the cDNA was packaged in vitro in lambda phage using Gigapak III Gold (Stratagene, available from Toyobo) to prepare a phage library.

Using the antibody obtained in Example 1 (2) as a probe, the phage library was screened for the antitumor gene. Twenty-one phages were tested positive. The procedure is as follows:

The concentration of the library was determined with titer. About 2,000 to 20,000 phages and 600µl E. coli (XL1-Blue) were plated in 150 mm NZYM culture plates together with 6ml NZYM Top Agar (0.7%). They were incubated at 42°C for 3-4 hours until plaques developed to suitable sizes of about 1 mm. Then, a 130-140 mm nitrocellulose membrane soaked with 10 mM IPTG was placed on each plate, and incubation was continued at 37°C for 3 hours. After the plates were cooled at 4°C for 1 hour or more, the nitrocellulose filters were removed from the plates, and shaken in TBS-T buffer solution containing 3% skim milk.

Next, the filters were soaked in the buffer solution of the primary antibody (Example 1 (2)), and gently shaken in TBS-T buffer solution containing 3% skim milk. The filters were then soaked in the buffer solution of secondary antibody conjugated to alkali phosphatase (AP), and washed with TBS-T buffer solution. After they were washed with alkali phosphatase (AP) buffer solution, positive phages were detected.

The resulting positive phages were transformed with SOLR strains (Stratagene) by in vivo excision, using ZAP-cDNA Synthesis Kit (available from Toyobo) according to a manufacturer's manual.

Plasmid pTS18 as shown in Fig. 1 was obtained from the transformants. Plasmid pTS18 (containing the cDNA sequence in SEQ ID NO. 1) was used in Example 3 as an expression vector.

The resulting pTS18 was deleted by using Exo/Mung DNA Sequencing System (Stratagene), blunted at both terminals, and ligated with self-DNA (Fig. 2). Next, E. coli JM109 (Toyobo) was transformed with the deleted plasmid DNA. The nucleotide sequences of the portions of the gene into which deletion mutation was introduced were completely determined using ABI PRISM Cycle Sequencing Kit (Parkin Elmer) both on the sense and anti-sense chains.

The determined partial sequences were used to establish the complete amino acid sequence and cDNA sequence (SEQ ID NO.2) of the antitumor protein. A deduced molecular weight was about 62 kDa. The amino acid sequence on the N terminal (SEQ ID NOS.3 and 4) agreed with the amino acid sequence 2-30 and the amino acid sequence 2-58 in SEQ ID NO.1.

Also, the sequences of the peptide fragments (SEQ ID NOS.5-18) agreed with the amino acid sequence in SEQ ID NO.1 as follows:

SEQ ID NO.5: 59-77 in SEQ ID NO.1;
SEQ ID NO.6: 89-149 in SEQ ID NO.1;
SEQ ID NO.7: 150-178 in SEQ ID NO.1;
SEQ ID NO.8: 179-209 in SEQ ID NO.1;
SEQ ID NO.9: 210-267 in SEQ ID NO.1;
SEQ ID NO.10: 268-297 in SEQ ID NO.1;
SEQ ID NO.11: 298-355 in SEQ ID NO.1;
SEQ ID NO.12: 356-406 in SEQ ID NO.1;
SEQ ID NO.13: 407-436 in SEQ ID NO.1;
SEQ ID NO.14: 437-486 in SEQ ID NO.1;
SEQ ID NO.15: 487-521 in SEQ ID NO.1;

SEQ ID NO.16: 522-554 in SEQ ID NO.1;
SEQ ID NO.17: 555-566 in SEQ ID NO.1;
SEQ ID NO.18: 78-99 in SEQ ID NO.1.

These peptide fragments are useful as antigens for obtaining an antibody against the antitumor protein which can be used in a method for screening and purifying an antitumor protein.

Example 3 Production of Antitumor Protein (1)

Competent cells (JM109 strain; Toyobo) stored at -80°C were melted, and 100 µl of the cells was transferred to Falcon tube (code 2059). It was mixed with deleated clones of pTS18 (Example 2) and allowed to stand in iced water for 30 min. After exposed to a thermal shock (42°C) for 30 s, it was cooled in ice for 2 min. After 900 µl SOC culture was added, it was incubated at 37°C for 1 hour with shaking. The cells were then planted in an LB/Amp plate in an appropriate amount, and incubated overnight at 37°C. A colony having an area of a platinum ring that appeared on the plate was transplanted to a liquid LB culture (containing Amp), and incubated at 37°C until absorption at 660 nm (Abs660) increased to about 0.2. Then, after IPTG was added to a final concentration of 10 mM, the culture was incubated until Abs660 increased to about 1.

The cells were suspended in the extract (50 mM Tris-HCl, pH 7.5) used in Example 1 (1), which contained PI, and ultrasonically destroyed. After the extract (50 mM Tris-HCl) was centrifuged, the supernatant was recovered in the eluate via affinity chromatography (CNBr-activated Sepharose 6MB resin; Pharmacia) binding the antibody described in Example 1 (2).

The eluate was analyzed by SDS-PAGE combined with Western blotting using the antibody described in Example 1 (2). The result showed that the protein according to the present invention was expressed in the host cell.

Example 4 Production of Antitumor Protein (2)

(1) Preparation of expression vector pET-28a

A DNA fragment encoding the antitumor protein was generated by polymerase chain reaction (PCR) using plasmid pTS18 (10 ng)(Example 2) as a template DNA. PCR reaction was carried out using reagents packaged in a commercialy available kit (TAKARA Co.) and the following primers (5 pmole, each) in accordance with a manufacturer's manual.

Primer 1:GAGAGACCATGGGGTATCGTCTTTCC (SEQ ID NO.19)

Primer 2:GAGAGAGGATCCGGAGACGCCAAGGAT (SEQ ID NO.20)

After the PCR reaction, the product was digested by NcoI and BamHI. The resulting fragment (0.1 μg) was ligated into the NcoI/BamHI site of pET-28a (0.5 μg) (Novagen).

The resulting DNA construct was introduced into competent cells (E. Coli, DH5α and JM109 strains; Toyobo). The plasmid DNA which was harvested from the transformed cells was introduced into competent cells (BL21 (DE3) strain; Novagen).

(2) Preparation of expression vector pET-28b

A DNA fragment encoding the antitumor protein was prepared by digesting plasmid pTS18 (Example 2) by EcoRI and XhoI and collecting EcoRI/XhoI fragments. The resulting fragments (0.1 μg) were ligated into the EcoRI/XhoI site of pET-28b (0.5 μg)(Novagen).

The resulting DNA construct was introduced into competent cells (E. Coli, DH5α and JM109 strains; Toyobo). The plasmid DNA which was harvested from the transformed cells was introduced into competent cells (BL21 (DE3) strain; Novagen).

(3) Expression of antitumor protein gene

One loopful of the transformed cells, BL21 (DE3) strain having pET-28a and BL21 (DE3) strain having pET-28b, obtained as described in Example 3 (1) and (2) were inoculated on 1 ml of NZYM medium containing 50 μg/ml of Kanamycin and preincubated at 37℃ overnight. 100 μl taken from the cultured medium was inoculated on 10 ml of NZYM medium containing 50 μg/ml of kanamycin and incubated at 25℃ until Abs600 increased to about 0.4. After IPTG was

added to a final concentration of 1.0 mM, the culture was incubated for 24 hours.

The cells were harvested from the culture medium, suspended in the extract (25 mM Tris-HCl, pH 7.0) used in Example 1 (1) containing PI, and ultrasonically destroyed.

After the extract (25 mM Tris-HCl, pH 7.0) was centrifuged, the precipitate was recovered. The precipitate was analyzed by SDS-PAGE. A single band was observed on the position of 65 kDa.

The precipitate was also analyzed by Western blotting using the antibody described in Example 1 (2). An immunoreactive band was observed at the same position as that observed on the SDS-PAGE gel. This result showed that the gene of the antitumor protein was expressed in the host cells.